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Original Article

Rhizobacteria activates (+)-δ-cadinene synthase genes and induces systemic resistance in cotton against beet armyworm (*Spodoptera exigua*)

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ABSTRACT

Gossypol is an important allelochemical produced by the subepidermal glands of some cotton varieties and important for their ability to respond to changing biotic stress by exhibiting antibiosis against some cotton pests. Plant growth-promoting rhizobacteria (PGPR) are root-colonizing bacteria that increase plant growth and often elicit defence against plant pathogens and insect pests. Little is known about the effect of PGPR on cotton plant–insect interactions and the potential biochemical and molecular mechanisms by which PGPR enhance cotton plant defence. Here, we report that PGPR (*Bacillus* spp.) treated cotton plants showed significantly higher levels of gossypol compared with untreated plants. Similarly, the transcript levels of the genes (i.e. (+)-δ-cadinene synthase gene family) involved in the biosynthesis of gossypol were higher in PGPR-treated plants than in untreated plants. Furthermore, the levels of jasmonic acid, an octadecanoid-derived defence-related phytohormone and the transcript level of jasmonic acid responsive genes were higher in PGPR-treated plants than in untreated plants. Most intriguingly, *Spodoptera exigua* showed reduced larval feeding and development on PGPR-treated plants. These findings demonstrate that treatment of plants with rhizobacteria may induce significant biochemical and molecular changes with potential ramifications for plant–insect interactions.

Key-words: CAD; gene expression; gossypol; jasmonic acid (JA); larval feeding; PGPR.

INTRODUCTION

The ability of plants to actively defend themselves with inducible and constitutive mechanisms has been extensively demonstrated in many systems (Chen *et al.* 2008; Mao *et al.* 2007a; Sarwar *et al.* 2005; Zebelo *et al.* 2011). Host plant resistance is an important component of integrated pest management (IPM), which can be triggered by biotic (pathogen and non-pathogen) or abiotic elicitors (like insect and pathogen originated elicitor chemicals). Cotton plants have numerous inducible defence mechanisms that are important for their ability to respond to changing biotic stress, including the synthesis of volatile terpenes (McAuslane & Alborn 1998; Morawo & Fadamiro 2014; Pare & Tumlinson 1997; Sobhy *et al.* 2014), phytoalexins, tannins, tyloses, pathogenesis-related proteins, as well as lignification, and the release of active oxygen species (Bell 1981; Townsend *et al.* 2005a).

Gossypol, a phenolic sesquiterpenoid aldehyde, is an important allelochemical produced by the subepidermal glands of some cotton varieties and exhibits antibiosis against some cotton pests (Wu *et al.* 2010). Du *et al.* (2004) found a positive correlation between gossypol level of various glanded cotton varieties and the abundance of *Aphis gossypii*. In glanded cotton varieties, gossypol and its precursor terpene aldehyde are inducible and developmentally regulated (Townsend *et al.* 2005a; Xu *et al.* 2004). Zhang *et al.* (2011) demonstrated that exogenous application of jasmonic acid (JA) to cotton plants resulted in increased levels of gossypol and that mealybug *Phenacoccus solenopsis* that fed on JA-treated cotton plants showed reduced growth and development. In the course of plant defence against herbivores in which various phytohormones are involved, JA is the key phytohormone related to plant defence against herbivores (Rani & Jyothsna 2010; War *et al.* 2011). JA is derived from linolenic acid through octadecanoid pathway and accumulates upon herbivory in plant tissues (Zhang *et al.* 2008). JA has been repeatedly shown to be the most important mediator of plant–herbivore interactions and to be responsible for volatile organic compound (VOC) activation including the synthesis terpenes (Baldwin 2010; Degenhardt *et al.* 2010; Dicke & Baldwin 2010).

The biosynthesis of sesquiterpene compounds, in general, takes place in the cytosol with farnesyl diphosphate as a common substrate. Farnesyl diphosphate synthase catalyses the formation of precursors for many different types of sesquiterpenes (Bohlmann *et al.* 1998; Croteau 1993). In cotton, (+)-δ-cadinene synthase (CAD) catalyses the cyclization of farnesyl diphosphate to (+)-δ-cadinene via a nerolidyl diphosphate intermediate and is the first step in the biosynthesis of gossypol and its precursor terpene aldehyde (Alchanati *et al.* 1998; Essenberg *et al.* 1985; Zhou *et al.* 2013). The CAD is encoded by a gene family, which has been divided into two subfamilies

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CAD1-A and CAD1-C (*Cdn1-C1*, *Cdn1-C14*, *Cdn1-A* and *Cdn1-C3*), based on sequence similarities. The diploid genome of *Gossypium arboreum* contains about six members of CAD1-C and a single copy of CAD1-A (Townsend *et al.* 2005a). The CAD1 transcripts increase dramatically along with the seed maturation in association with an increase in sesquiterpene cyclase activities and subsequently the accumulation of gossypol (Meng *et al.* 1999). CAD1 enzyme and transcripts are induced in cotton plants infected with the fungal pathogen, *Verticillium dahliae* and in cotton suspension cultures treated with *V. dahliae* elicitors (Chen *et al.* 1995; Townsend *et al.* 2005a). These studies revealed that CAD1 is the key enzyme in gossypol formation, which is developmentally regulated and also induced by pathogen infection in cotton.

Plants interact with beneficial soil microbes, such as plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi, that confer beneficial effects including increased plant growth and enhanced tolerance to abiotic stress (Dimkpa *et al.* 2009; Kloepper *et al.* 2004). Some beneficial microbes also induce systemic resistance (ISR) against microbial pathogens and herbivorous insects (Gange *et al.* 2003; Murphy *et al.* 2003; Pineda *et al.* 2013; Pineda *et al.* 2010; Van Oosten *et al.* 2008; Zamioudis & Pieterse 2012).

One of the pioneering studies that showed that PGPR can elicit ISR against insects was conducted using PGPR strain INR-7 (*Bacillus pumilis*). In a series of greenhouse and field experiments, Zehnder *et al.* (1997a) demonstrated that treating cucumber (*Cucumis sativus* L.) plants with INR-7 increased plant growth and significantly reduced the population of the striped cucumber beetle, *Acalyma vittatum* Fabricius (Coleoptera: Chrysomelidae), and the spotted cucumber beetle, *Diabrotica undecimpunctata* Howardi (Coleoptera: Chrysomelidae). Interestingly, the reduced number of beetles on PGPR-treated plants was correlated with reduced production of curcubitacin, a secondary plant metabolite and insect-feeding stimulant (Zehnder *et al.* 1997b). Aside from these initial studies, little is known about the effects and mechanisms by which PGPR directly or indirectly mediate plant defence to herbivore damage (Pineda *et al.* 2010).

To establish predictable patterns in PGPR-induced systemic resistance against insects, more comprehensive studies that combine ecological, biochemical and molecular approaches are needed. In addition, recent studies have showed that mixtures of PGPR can provide synergistic ISR activity against a broader range of plant pathogens compared with individual PGPR strains (Domenech *et al.* 2006; Jetiyanon & Kloepper 2002; Ryu *et al.* 2007). The present study utilizes ecological, biochemical and molecular approaches to investigate the effects and mechanisms of PGPR strains or mixtures on plant–insect interactions. We used a model system consisting of cotton (*Gossypium hirsutum*) and *Spodoptera exigua* (a generalist larval pest of cotton) to investigate the effect of treatment of cotton plants with single strain or mixture of strains (blends) of PGPR (*Bacillus* spp.) on plant chemistry and plant–insect interactions. The central hypothesis tested was that PGPR treatment of cotton plants will induce direct plant defence mechanisms against herbivory via increased expression of genes involved in gossypol biosynthesis. Firstly, we quantified the level of phytohormone JA and gossypol in PGPR-treated and untreated cotton plants. Secondly, the transcript levels of JA responsive genes and the genes involved in gossypol biosynthesis were compared between PGPR-treated and untreated plants. Finally, bioassays were performed to evaluate feeding and development of *S. exigua* larvae on the various treatments.

MATERIALS AND METHODS

Plant growth and plant growth-promoting rhizobacteria treatment

Cotton plants (*Gossypium hirsutum*, Var. Max-9, All-Tex Seed, Inc. Levelland, TX, USA) were grown in growth chambers under controlled conditions (23 ± 2 °C, 60 ± 5% RH and a photoperiod of 16:8 h light:day) using sterilized sunshine potting mixture #8 (SunGro Horticulture, Agawam, MA, USA). PGPR treatments were applied at seeding (1 mL/seed) as aqueous spore suspensions (1×10^7spores/mL). The bacterial mixtures were prepared by mixing equal volumes (2.5 mL) of spore suspensions of each strain to a final concentration of 1×10^7spores/mL. Booster applications of PGPR were applied weekly from 1 to 6 weeks after planting by pipetting 1 mL of vegetative PGPR suspension at 1×10^9cfu/mL around the rhizosphere of growing plants. For the booster application, the bacterial mixtures were prepared by mixing equal volumes (2.5 mL) of vegetative suspensions of each strain to a final concentration of 1×10^9cfu/mL. The PGPR strains used in this experiment were obtained from the PGPR culture collection at the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains (single or blend) have plant growth-promoting effect and enhance plant defence against disease-causing pathogens and insect pests (Calvo 2013; Enebak *et al.* 1998; Jetiyanon *et al.* 2003; Kokalis-Burelle *et al.* 2003; Murphy *et al.* 2003; Ryu *et al.* 2007; Sato *et al.* 2000; Zehnder *et al.* 1997a; Zehnder *et al.* 1997b). The following PGPR strains were evaluated: (1) strain INR-7 (AP 18: *Bacillus pumilis*); (2) blend 8, containing four strains of bacilli (AP-188: *Bacillus amyloliquefaciens* subsp. *Plantarum*, AP-209: *Bacillus mojavensis*, AP-217: *Bacillus solisalsi*, AP-218: *Bacillus amyloliquefaciens* subsp. *plantarum*); and (3) blend 9, containing four strains of bacilli (AP-136: *Bacillus amyloliquefaciens* subsp. *plantarum*, AP-188: *Bacillus amyloliquefaciens* subsp. *plantarum*, AP-219: *Bacillus amyloliquefaciens* subsp. *plantarum*, AP-295: *Bacillus amyloliquefaciens* subsp. *plantarum*). These single and blended strains have showed plant growth promotion in cotton plants (Fadamiro *et al.* 2012).

Insect rearing

Spodoptera exigua eggs purchased from Benzon Research (Carlisle, PA, USA) were used to start laboratory colonies at Auburn University (Auburn, AL, USA). Larvae were fed a laboratory-prepared pinto bean diet at 25 ± 1 °C, 75 ± 5% relative humidity and 14:10 h (light:day) photoperiod. Eggs from the F2 generation of the colony were used for survival and development test. Fifteen third instar larvae per sample and

pattern wheel were used to make herbivore damage and mechanical damage in JA, gossypol and transcript level quantification experiments, respectively.

Jasmonic acid extraction and quantification

Cotton plants were grown as described earlier without PGPR treatments. In order to measure the level of JA in real time, 4 weeks after planting, cotton plants were subjected to the following treatments: (1) untreated (controls); (2) treated with PGPR [INR7, blend 8 (B8) and blend 9 (B9)]; (3) *S. exigua* larvae damaged (HD); and (4) mechanically damaged (MD). Cotton leaves from untreated, B8, B9, INR7, HD and MD were collected 1, 2, 3 and 4 d after their respective treatments, frozen in liquid nitrogen and kept in −80 °C until use. JA was extracted as reported by Occhipinti *et al.* (2011). The content of JA was determined by comparing retention times and mass spectra of standard solutions with a linear gradient in reversed phase-chromatography (Luna C18, 3.0 × 150 mm, 3.0 μm; Phenomenex, Torrance, CA, USA), and further analyses with samples were analysed on a Waters ESI-MS (Q-Tof Premier, Waters). Samples were isocratically eluted from a 150 × 3.9 mm i.d. Waters (4 μm) C18 Novapak column. The mobile phases were 95% acetonitrile and formic acid. At the flow rate of 0.15 mL min^{-1}, total run time was 10 min. The signal was monitored at 272 nm. Data collection and integration were performed using the Waters Empower software (Waters 2489, Milford, MA, USA). H2-JA (TCI-Europe) was used as internal standard, and precursor ions were detected in negative mode by multiple reaction monitoring [M-H] 209 and 211, for JA, and H2-JA, respectively. The reported data are the mean values of at least three biological replicates and five technical replicates. The data were analysed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer HSD multiple comparison test at a significance level of $P < 0.05$.

Gossypol extraction and quantification

Cotton plants were grown as described above without PGPR treatments. In order to measure the level of gossypol in real time, 4 weeks after planting, cotton plants were subjected to the following treatments: (1) untreated (controls); (2) treated with PGPR [INR7, blend 8 (B8) and blend 9 (B9)]; (3) *S. exigua* larvae damaged (HD); and (4) mechanically damaged (MD). Cotton leaves from untreated, B8, B9, INR7, HD and MD were collected 1, 2, 3 and 4 d after their respective treatments, frozen in liquid nitrogen and kept in −80 °C until use. Gossypol was extracted as previously described by Zhang *et al.* (2011) with some modification. The samples were ground by using liquid nitrogen in a mortar. Lyophilized leaf samples (100 mg) were weighed into centrifuge tubes and extracted by ultrasonification (3 min) in a solvent (acetonitrile/water/phosphoric acid = 80:20:0.1; 10 mL). The samples were centrifuged (3 min at 2800 *g*), and an aliquot of the supernatant was transferred directly into a vial. Standard gossypol (95% purity; Sigma-Aldrich, USA) was dissolved in the extraction solvent. Standard curves were obtained for gossypol with concentrations in the range of 5–80 μg mL^{-1} in five-step increments. Three samples were collected for each treatment. Samples were analysed on a Waters ESI$^-$-MS (Q-Tof premier, Waters). Samples were isocratically eluted from a 150 × 3.9 mm i.d. Waters (4 μm) C18 Novapak column. The mobile phases were 95% acetonitrile and formic acid. At the flow rate of 0.15 mL min^{-1}, total run time was 10 min. The signal was monitored at 272 nm. Data collection and integration were performed using the Waters Empower software (Waters 2489, Milford, MA). The reported data are the mean values of at least three biological replicates and five technical replicates. The data were analysed by one-way ANOVA followed by the Tukey–Kramer HSD multiple comparison test at a significance level of $P < 0.05$.

Total RNA isolation and cDNA synthesis

Cotton plants were grown as described previously without PGPR treatments. In order to measure the transcript levels of defence-related genes in real time, 4 weeks after planting, cotton plants were subjected to the following treatments: (1) untreated (controls); (2) treated with PGPR [INR7, blend 8 (B8) and blend 9 (B9)]; (3) *S. exigua* larvae damaged (HD); and (4) mechanically damaged (MD). Cotton leaves from untreated, B8, B9, INR7, HD and MD were collected 1 d after their respective treatments. Three biological replicates and seven technical replicates were analysed as follows. Cotton leaves sampled immediately frozen in liquid nitrogen and kept in −80 °C freezer until use. Frozen samples were ground to a fine powder in liquid nitrogen with a pestle and mortar. Total RNA was extracted from 100 mg of each leaf sample using Spectrum^{TM} plant total RNA kit (Sigma Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. RNA concentration and purity were determined by using a NanoDropTM Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, USA), and the integrity of RNA was also assessed by 1% agarose gel electrophoresis and ethidium bromide staining. The presence of contaminant DNA in the RNA samples was verified by PCR using specific primers of a known gene and gel electrophoresis analysis. No fragments of genomic DNA were identified in any samples tested in this work. First- strand cDNA was synthesized from 200 ng RNA using a First-Strand cDNA Synthesis, which employed a Goscrpit^{TM} reverse transcription system Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Real-time PCR

To quantify the transcript levels, JA responsive genes (*GhAOS*, *GhLOX1* and *GhOPR3*) and CAD family genes (i.e. *Cad1-A*, *Cdn1-C1*, *Cdn1-C14* and *Cdn1-C3*; Table 1) in cotton leaves from the various plant treatments {i.e. untreated; PGPR-treated [INR7, blend 8 (B8) and blend 9 (B9)]; *S. exigua* damaged (HD); and mechanically damaged (MD)} were measured by quantitative RT-PCR. The real-time PCR was carried out on an ABI 7500 Real-time PCR system (Life Technologies, Carlsbad, CA, USA) with

Table 1. Primer sequences used for qrt-PCR

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	GenBank accession numbers
Histone (H3)	GAAGCCTCATCGATACCGT	CTACCACTACCATCATGGC	AF024716
GhLOX1	GCCAAGGAGAGCTTCAAGAAT	TAGGGGTACTTGGCAGAACCT	AF361893
GhAOS	ATCATGTAATCCCCGAGTTCC	CCAGCTTGATCGTTAGCTGTC	KM265129
OPR3	ATGTGACGCAACCTCGTTATC	CCGCCACTACACATGAAAGTT	FB505932
Cad1-C1	TTTGCATAGGAAAGAGCTA	CTCTATTGCTGAGCAATCAT	U23206
Cad1-A	ATAAGGATGAAATGCGTCC	GAAGCTTGGTAAAGTTCCA	Y18484
Cdn1-C14	AACTCAAAAACGCCACCAAC	TAGTCGGAATCGAAGGGATG	U23205
Cdn1-C3	AACTCAAAAACGCCACCAAC	TAGTCGGAATCGAAGGGATG	AF174294

a 96-well rotor. The amplification reactions were performed with 25 μL of mixture consisting of 12.5 μL of PerfeCTA® SYBR® Green Fastmix®, ROX qPCR Master Mix (Qunita Biosciences, Inc, Gaithersburg, MD, USA), 0.5 μL of cDNA and 100 nM primers (Integrated DNA Technologies, Coralville, IA, US). Relative RNA levels were calibrated and normalized with the level of stable housekeeping gene histone (H_3) ribosomal mRNA. PCR conditions were determined by comparing threshold values in dilution series of the RT product followed by non-template control for each primer pair. Relative expression levels of genes were calculated by using the Pfaffl method (Pfaffl 2001). A suitable melt curve analysis was always performed. To confirm the specificity of the primers, qrtPCR amplicons were sequenced using Life Technologies POP 7 technology for DNA sequencing (Carlsbad, CA, USA). The sequencing of amplicons confirmed that single products corresponding to the contigs that were used for primer design (Supporting Information Table S1). All genes were similar to known nucleotide sequences using BLAST with a score value higher than 100 and identity ranging from 90 to 97%.

Effect of plant growth-promoting rhizobacteria treatment on survival and development of *Spodoptera exigua* larvae

Spodoptera exigua eggs were placed in a 10 cm diameter plastic Petri dish and provided with 6-week-old cotton plants treated with PGPR (INR7, blend 8 and blend 9) at seeding and untreated ones. The bioassay was performed in an incubator (25 ± 1 °C, 75 ± 5% relative humidity and with a light phase of 16 h). After larval hatch, the fate of a single neonate larva on each leaf was followed until dead or pupation. To avoid desiccation, the cotton leaf petiole was immersed in a 1.5 mL Eppendorf tube containing distilled water. Larval weight was recorded every 3 d, and development and mortality were monitored daily until pupation. Pupal weight was determined within 24 h of pupation. The study included three sets of independent experiments with 40 insects per treatment. The data were analysed by using one-way ANOVA followed by the Tukey–Kramer HSD multiple comparison ($P < 0.05$).

RESULTS

Plant growth-promoting rhizobacteria treatment affects levels of jasmonic acid

Generally, the level of the phytohormone, JA in cotton plants treated with PGPR remained high throughout the experimental period, unlike in cotton plants damaged by *S. exigua* in which the JA level reached its peak in the first 24 h of the experiments and decreased throughout the rest of the experimental period. On the first day of the experiment, the amount of JA in PGPR-treated plants was not significantly different from that found in cotton plants with *S. exigua* damage. Both were significantly higher than the amount found in untreated plants and mechanically damaged plants. On the second day, the level of JA in PGPR-treated plants was significantly higher than in *S. exigua* larva damaged plants, untreated plants and mechanically damaged plants (Fig. 1a). The levels of JA progressively increased on days 3 and 4 in PGPR-treated plants but remained low in other treatments.

Plant growth-promoting rhizobacteria treatment induces increased level of gossypol in cotton plants

No significant differences in gossypol levels were recorded between untreated and PGPR-treated cotton plants up to 2 d after PGPR treatment. However, the amount of gossypol in PGPR-treated plants was significantly higher than in untreated plants 4 d after PGPR treatment (Fig. 1b). On the first day of the experiment (D1), the amount of gossypol in cotton plants damaged by *S. exigua* larvae was significantly higher than in PGPR-treated plants. The amount of gossypol was not significantly higher in PGPR-treated plants than that found in untreated plants and mechanically damaged plants. On the second day (D2), the amount of gossypol in PGPR-treated plants was not significantly different from that found in cotton plants damaged by *S. exigua* larvae. Both were significantly higher than the amount found in untreated plants and mechanically damaged plants (Fig. 1b). The level of gossypol was higher on day 3 (D3) than on day 2 (D2) in PGPR-treated plants and remained low in plants with *S. exigua* larva infestation and mechanically damaged plants. On the fourth day (D4), the level of gossypol was higher than the second

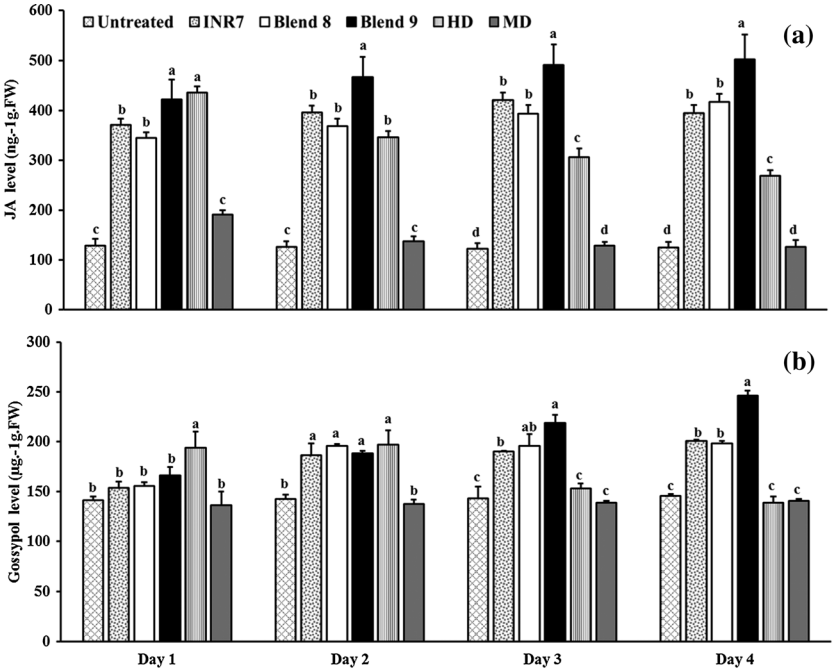

Figure 1. Jasmonic acid (JA) (a) and gossypol (b) levels in cotton leaves from untreated, plant growth-promoting rhizobacteria (PGPR) INR7-treated, PGPR blend 8-treated, PGPR blend 9-treated, *Spodoptera exigua* larval-damaged (HD) and mechanically damaged (MD) plants 1, 2, 3 and 4 d after PGPR treatment. Data indicate means (±SE) of JA (ng. − 1 g.FW) and gossypol (μg. − 1 g.FW) levels on cotton leaves of three replicates. Significant differences among different treatments are indicated by different letters ($P < 0.05$, $n = 3$ analysis of variance, Tukey–Kramer HSD multiple comparison test).

day (D2) and third day (D3) in PGPR-treated plants and remained lower in PGPR-untreated plants and plants damaged by *S. exigua* larvae (Fig. 1b). The level of gossypol was significantly higher in PGPR blend 9-treated plants than PGPR blend 8 and INR7-treated plants on the D3 and D4.

Plant growth-promoting rhizobacteria treatment induces defence-related gene expression in treated plants

PGPR treatment and *S. exigua* damage were found to increase the transcript levels JA responsive genes, *GhAOS*, *GhLOX1* and *GhOPR3*. The transcript levels of *GhAOS*, *GhLOX1* and *GhOPR3* were increased in PGPR blend 9 treated plants by 2.8, 2.5 and 2.5 fold compared to untreated control, respectively (Fig. 2). Transcript levels of *GhLOX1* gene increased by 2.5-folds after being treated with PGPR blend 8 and by 2.3-folds after treatment with PGPR strain INR7. The expressions of JA responsive genes were also significantly higher in *S. exigua* larvae damaged plants compared with untreated control (Fig. 2).

Similarly, PGPR treatment and *S. exigua* damage were found to increase the transcript levels of most of the genes involved in the biosynthesis of gossypol, such as *CAD1-C1*, *Cdn1-A*, *Cdn1-C14* and *Cdn1-C3*. The transcript levels of *CAD1-C1*, *Cdn1-A*, *Cdn1-C14* and *Cdn1-C3* were increased in PGPR blend 9 treated plants by 2.0, 2.8, 2.2 and 2.5-fold compared with untreated control, respectively (Fig. 2). The transcript levels of CAD1 family genes such as *CAD1-C1*, *Cdn1-A*, *Cdn1-C14* and *Cdn1-C3* were increased in *S. exigua* larvae damaged plants by 1.1, 2.4, 1.0 and 2.7-fold compared with untreated control, respectively (Fig. 2). The transcript level of *Cdn1-C14* gene was higher in PGPR INR7 treated plants (twofold) than in untreated control and mechanically damaged plants (Fig. 2). Generally, PGPR blend 9 treated plants induced higher transcript levels of CAD1 genes than PGPR blend 8 and INR7-treated plants. Most of CAD1 genes failed to show increased transcript level in untreated control plants and mechanically damaged plants.

Plant growth-promoting rhizobacteria treatment impacts survival and development of *Spodoptera exigua* larvae

Spodoptera exigua larvae that fed on untreated cotton plants had greater weight (Fig. 3), a lower mortality rate (Fig. 4) and a higher pupation rate (Fig. 5) compared with larvae that fed on PGPR-treated plants. Larval mortality was significantly lower on untreated plants compared with the three PGPR treatments. For instance, fourth-instar larval mortality (77%) was highest on blend 9-treated plants compared with 40% mortality on untreated plants (Fig. 4).

DISCUSSION

The results of this study showed that the beneficial rhizobacteria can induce biosynthesis of insecticidal compound such as gossypol with significant ramifications for

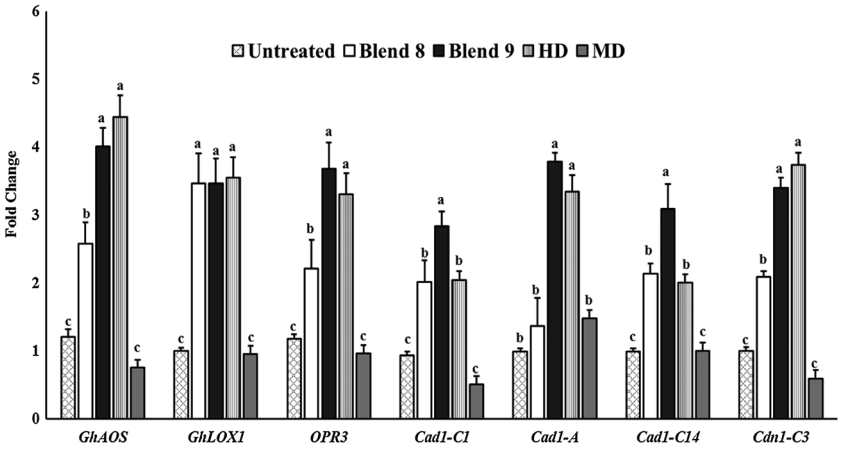

Figure 2. Quantitative real-time PCR (qRT-PCR) results are shown as fold changes (±SE). Different letters indicate significant differences after 2 d of the treatment with plant growth-promoting rhizobacteria (PGPR) strains of INR7, blend 8, blend 9, herbivore damage (HD) and mechanical damage (MD). Jasmonic acid responsive genes (*GhAOS*, *GhLOX1* and *GhOPR3*) and (+)-*δ*-cadinene synthase (CAD1) gene family include *Cad1-C1*, *Cad1-A*, *Cad1-C14 and Cdn-C3* ($P < 0.05$; Tukey–Kramer HSD multiple comparison test, $n = 4$).

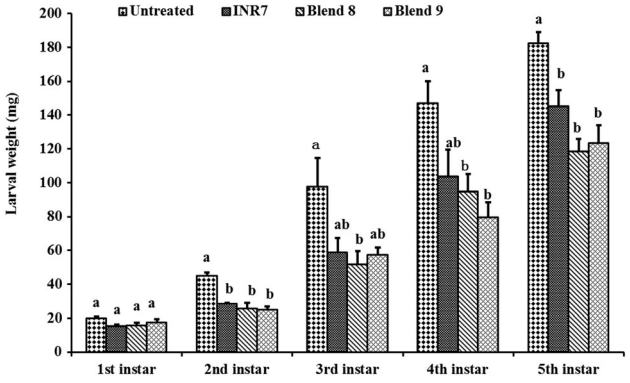

Figure 3. Effect of plant growth-promoting rhizobacteria treatment on weight of developing *Spodoptera exigua* larvae. Means (±SE) within each instar category with different letter are significantly different ($n = 40$, $P < 0.05$, Tukey–Kramer HSD multiple comparison test).

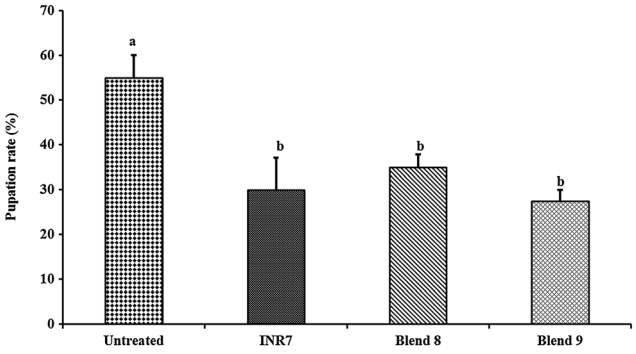

Figure 5. Effect of plant growth-promoting rhizobacteria treatment on *Spodoptera exigua* larvae pupation rate. Means (±SE) with different letter are significantly different ($n = 40$, $P < 0.05$, Tukey–Kramer HSD multiple comparison test).

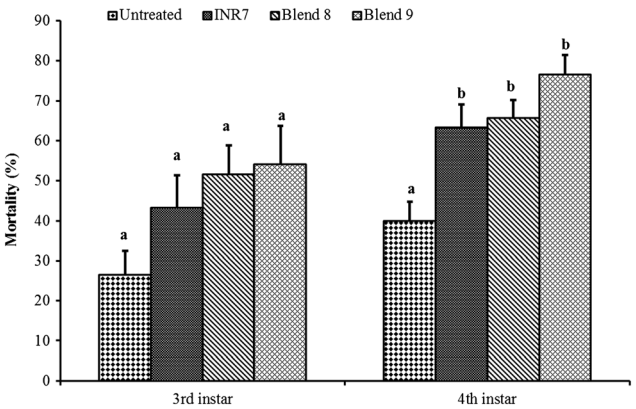

Figure 4. Effect of plant growth-promoting rhizobacteria treatment on *Spodoptera exigua* larval mortality. Means (±SE) within each instar category with different letter are significantly different ($n = 40$, $P < 0.05$, Tukey–Kramer HSD multiple comparison test).

plant–insect interactions. Specifically, higher levels of gossypol were detected in cotton plants treated with certain strains or mixtures of PGPR (*Bacillus* spp.) compared with untreated plants. Furthermore, the transcript levels of genes that encode gossypol biosynthesis were higher in PGPR-treated cotton than untreated plants. The increased level of gossypol in PGPR-treated plants might correlate with the increased level of JA observed in PGPR-treated plants. Consequently, *S. exigua* larvae showed reduced herbivory and development on PGPR-treated plants compared with untreated plants. Thus, treatment with PGPR can induce biochemical and molecular changes in plants with important ramifications for plant–insect interactions. Our findings are consistent with the results of some recent studies that showed that treatment of plants with *Pseudomonas* spp. induced physiological and biochemical changes in certain plants, with negative consequences for herbivorous insects (Pineda *et al*. 2013; Pineda *et al*. 2010; Van Oosten *et al*. 2008).

Jasmonic acid is an ubiquitous plant hormone known to mediate defence responses in plants (Browse 2005). High level of JA was recorded in PGPR-treated plants. Previous studies showed that PGPR trigger induced ISR via JA signal-transduction pathway (Pieterse *et al.* 2009; Pineda *et al.* 2012; Pozo *et al.* 2008; Van Oosten *et al.* 2008). Opitz *et al.* (2008) reported that exogenous application of JA induces increased level of gossypol and other terpenoid production in cotton plants. Gossypol is an important allelochemical occurring in glanded cotton varieties that can be induced by exogenous JA application (Opitz *et al.* 2008).

High gossypol level expressed by some cotton varieties has been shown to affect feeding and development of certain insect pests (Mao *et al.* 2007b; Zhang *et al.* 2011). The data showed that gossypol level was elevated in PGPR-treated plants, which possibly render PGPR-treated plants unsuitable for *S. exigua* larval development. PGPR blend 9-treated plants showed increased level of gossypol compared with other treated plants throughout the experimental period, and this might explain a continuous elicitation of induced systemic resistance of the plant by the colonizing *Bacillus* spp. component of PGPR blend 9. It has been suggested that mixtures of PGPR strains with different mechanisms of interactions might more reliably benefit plants than would individual PGPR strains (Raupach & Kloepper 1998). Choudhary *et al.* (2007) described that plants have the ability to acquire an enhanced level of resistance to pathogens after exposure to biotic stimuli provided by many different PGPRs, and these associations with plant roots elicit a steady state of defence or ISR in plants.

To understand the underlying molecular mechanisms behind the observed increased levels of JA and gossypol in PGPR-treated plants, we investigated the expression of the putative cotton JA responsive genes and genes involved in the biosynthesis of gossypol. The expression of the putative cotton JA-related genes, *GhAOS*, *GhLOX1* and *GhOPR3*, showed general increases in transcript level in PGPR-treated plants. The expression of these JA responsive genes was previously shown to be induced in cotton plants exogenously treated with JA or methyl jasmoniate or both (Miyazaki *et al.* 2014). These JA responsive genes have been associated with induced responses against herbivorous insects in various plants such as lima bean (Arimura *et al.* 2000), cabbage (Zheng *et al.* 2007), tomato (Li *et al.* 2004) and cotton (Miyazaki *et al.* 2014). There is no isogenic mutant line for JA pathway available in cotton; however, our data clearly showed that the level of JA was high and significantly induced in PGPR-treated plants. The expression of genes involved in gossypol biosynthesis such as (+)-δ-cadinene synthase (CAD1) gene family (CAD1-*C1*, *Cdn1-A*, *Cdn1-C14* and *Cdn1-C3*) were higher in PGPR-treated plants and herbivore damage plants compared with untreated or mechanically damaged plants. CAD1 expression was previously shown to be induced in cotton infected with either the bacterial blight or verticillium wilt-causing pathogens (Townsend *et al.* 2005b).

For JA, gossypol and gene expression experiments, the use of 4-week-old plants that were grown in a well-controlled environment in order to minimize the effect of other factors that could affect level of JA, gossypol and gene expressions was helpful to test the real-time effect of PGPR in plant defence. For *S. exigua* feeding experiment, 6-week-old plants treated with PGPR at seeding were used for bioassay. Plants used for the feeding experiment were grown with minimal care to simulate the agro ecology in which the plants interact with insects.

Spodoptera exigua larvae that fed on untreated cotton plants showed better growth, lower mortality rate and higher pupation rate compared with larvae that fed on PGPR-treated plants. Larval mortality was highest on larvae that fed on PGPR blend 9-treated plants. Zehnder *et al.* (2000) reported similar results in which cucumber beetles showed reduced feeding damage on PGPR (INR7)-treated cucumber plants. Furthermore, pupation rates and pupal weight were significantly lower in insects that fed on PGPR-treated plants than in those that fed on untreated plants. These results showed that PGPR-treated plants do not optimally support larval feeding and development.

In summary, the results presented here suggest that PGPR treatment can enhance plant defence against insect herbivory by triggering ISR. Our data showed that PGPR treatment elicits the expression of JA-related genes and induces increased levels of JA that might lead to induction of transcripts of gossypol-related genes and increased levels of gossypol, which resulted in reduced herbivory by *S. exigua* larvae. In a related study, Fadamiro *et al.* (2012) reported that PGPR treatment of cotton plants resulted in increased emission of VOCs, including those known to affect insect behaviour. Thus, it is likely that the reduced herbivory on PGPR-treated plants recorded in this study is due to a combined effect of VOCs and gossypol. Studies are ongoing to determine the effects and mechanisms of PGPR-mediated interactions in other agro-ecosystems.

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REFERENCES

Alchanati I., Patel J.A.A., Liu J.G., Benedict C.R., Stipanovic R.D., Bell A.A., Cui Y.X. & Magill C.W. (1998) The enzymatic cyclization of nerolidyl diphosphate by delta-cadinene synthase from cotton stele tissue infected with *Verticillium dahliae*. *Phytochemistry* **47**, 961–967.

Arimura G., Ozawa R., Shimoda T., Nishioka T., Boland W. & Takabayashi J. (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature* **406**, 512–515.

Baldwin I.T. (2010) Plant volatiles. *Current Biology* **20**, R392–R397.

Bell A.A. (1981) Biochemical-mechanisms of disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **32**, 21–81.

Bohlmann J., Meyer-Gauen G. & Croteau R. (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 4126–4133.

Browse J. (2005) Jasmonate: an oxylipin signal with many roles in plants. *Plant Hormones* **72**, 431–456.

Calvo P. (2013) *Effect of Microbial Inoculation on Nitrogen Plant Uptake and Nitrogen Losses from Soil and Plant–Soil Systems*. Auburn University.

Chen H., Gonzales-Vigil E. & Howe G. (2008) Action of plant defensive enzymes in the insect midgut. In: *Induced Plant Resistance to Herbivory* (ed A. Schaller), pp. 271–284. Springer the Netherlands.

Chen X.Y., Chen Y., Heinstein P. & Davisson V.J. (1995) Cloning, expression, and characterization of (+)-delta-cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. *Archives of Biochemistry and Biophysics* **324**, 255–266.

Choudhary D., Prakash A. & Johri B.N. (2007) Induced systemic resistance (ISR) in plants: mechanism of action. *Indian Journal of Microbiology* **47**, 289–297.

Degenhardt D.C., Refi-Hind S., Stratmann J.W. & Lincoln D.E. (2010) Systemin and jasmonic acid regulate constitutive and herbivore-induced systemic volatile emissions in tomato, *Solanum lycopersicum*. *Phytochemistry* **71**, 2024–2037.

Dicke M. & Baldwin I.T. (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Science* **15**, 167–175.

Dimkpa C., Weinand T. & Asch F. (2009) Plant–rhizobacteria interactions alleviate abiotic stress conditions. *Plant, Cell and Environment* **32**, 1682–1694.

Domenech J., Reddy M.S., Kloepper J.W., Ramos B. & Gutierrez-Manero J. (2006) Combined application of the biological product LS213 with bacillus, pseudomonas or chryseobacterium for growth promotion and biological control of soil-borne diseases in pepper and tomato. *Biocontrol* **51**, 245–258.

Du L., Ge F., Zhu S. & Parajulee M.N. (2004) Effect of cotton cultivar on development and reproduction of *Aphis gossypii* (Homoptera: Aphididae) and its predator *Propylaea japonica* (Coleoptera: Coccinellidae). *Journal of Economic Entomology* **97**, 1278–1283.

Enebak S.A., Wei G. & Kloepper J.W. (1998) Effects of plant growth-promoting rhizobacteria on loblolly and slash pine seedlings. *Forest Science* **44**, 139–144.

Essenberg M., Stoessl A. & Stothers J.B. (1985) The biosynthesis of 2,7-dihydroxycadalene in infected cotton cotyledons – the folding pattern of the farnesol precursor and possible implications for gossypol biosynthesis. *Journal of the Chemical Society-Chemical Communications* 556–557.

Fadamiro H.Y., Kloepper J.W., Nangle K.W. & Ngumbi E.N. (2012) Inoculants including bacillus bacteria for inducing production of volatile organic compounds in plants. Google Patents.

Gange A.C., Brown V.K. & Aplin D.M. (2003) Multitrophic links between arbuscular mycorrhizal fungi and insect parasitoids. *Ecology Letters* **6**, 1051–1055.

Gershenzon. J. & Croteau R.B. (1993) Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes, and diterpenes. In: *Lipid Metabolism in Plants* (ed T. Moore), pp. 339–388. Raton, FL, CRC Press, Boca

Jetiyanon K., Fowler W.D. & Kloepper J.W. (2003) Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. *Plant Disease* **87**, 1390–1394.

Jetiyanon K. & Kloepper J.W. (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biological Control* **24**, 285–291.

Kloepper J.W., Ryu C.M. & Zhang S.A. (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**, 1259–1266.

Kokalis-Burelle N., Vavrina C.S., Reddy V.S. & Kloepper J.W. (2003) Amendment of muskmelon and watermelon transplant media with plant growth-promoting rhizobacteria: effects on seedling quality, disease, and nematode resistance. *Horttechnology* **13**, 476–482.

Li L., Zhao Y.F., McCaig B.C., Wingerd B.A., Wang J.H., Whalon M.E., Pichersky E. & Howe G.A. (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. *The Plant Cell* **16**, 126–143.

Mao J.Q., Burt A.J., Ramputh A.I., Simmonds J., Cass L., Hubbard K., … Arnason J.T. (2007a) Diverted secondary metabolism and improved resistance to European corn borer (*Ostrinia nubilalis*) in maize (Zea mays L.) transformed with wheat oxalate oxidase. *Journal of Agricultural and Food Chemistry* **55**, 2582–2589.

Mao Y.B., Cai W.J., Wang J.W., Hong G.J., Tao X.Y., Wang L.J., Huang Y.P. & Chen X.Y. (2007b) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology* **25**, 1307–1313.

McAuslane H.J. & Alborn H.T. (1998) Systemic induction of allelochemicals in glanded and glandless isogenic cotton by *Spodoptera exigua* feeding. *Journal of Chemical Ecology* **24**, 399–416.

Meng Y.L., Jia J.W., Liu C.J., Liang W.Q., Heinstein P. & Chen X.Y. (1999) Coordinated accumulation of (+)-delta-cadinene synthase mRNAs and gossypol in developing seeds of *Gossypium hirsutum* and a new member of the CAD1 family from *G. arboreum*. *Journal of Natural Products* **62**, 248–252.

Miyazaki J., Stiller W.N., Truong T.T., Xu Q., Hocart C.H., Wilson L.J. & Wilson I.W. (2014) Jasmonic acid is associated with resistance to twospotted spider mites in diploid cotton (*Gossypium arboreum*). *Functional Plant Biology* **41**, 748–757.

Morawo T. & Fadamiro H. (2014) Duration of plant damage by host larvae affects attraction of two parasitoid species (*Microplitis croceipes* and *Cotesia marginiventris*) to cotton: implications for interspecific competition. *Journal of Chemical Ecology* **40**, 1176–1185.

Murphy J.F., Reddy M.S., Ryu C.M., Kloepper J.W. & Li R.H. (2003) Rhizobacteria-mediated growth promotion of tomato leads to protection against *Cucumber mosaic virus*. *Phytopathology* **93**, 1301–1307.

Occhipinti A., Zebelo S.A., Capuzzo A., Maffei M. & Gnavi G. (2011) *Chrysolina herbacea* modulates jasmonic acid, cis-(+)-12-oxophytodienoic acid, (3R,7S)-jasmonoyl-l-isoleucine, and salicylic acid of local and systemic leaves in the host plant *Mentha aquatica*. *Journal of Plant Interactions* **6**, 99–101.

Opitz S., Kunert G. & Gershenzon J. (2008) Increased terpenoid accumulation in cotton (*Gossypium hirsutum*) foliage is a general wound response. *Journal of Chemical Ecology* **34**, 508–522.

Pare P.W. & Tumlinson J.H. (1997) Induced synthesis of plant volatiles. *Nature* **385**, 30–31.

Pfaffl M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**e45.

Pieterse C.M.J., Leon-Reyes A., Van der Ent S. & Van Wees S.C.M. (2009) Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* **5**, 308–316.

Pineda A., Soler R., Weldegergis B.T., Shimwela M.M., Van Loon J.J.A. & Dicke M. (2013) Non-pathogenic rhizobacteria interfere with the attraction of parasitoids to aphid-induced plant volatiles via jasmonic acid signalling. *Plant, Cell and Environment* **36**, 393–404.

Pineda A., Zheng S.J., van Loon J.J.A. & Dicke M. (2012) Rhizobacteria modify plant–aphid interactions: a case of induced systemic susceptibility. *Plant Biology* **14**, 83–90.

Pineda A., Zheng S.J., van Loon J.J.A., Pieterse C.M.J. & Dicke M. (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in Plant Science* **15**, 507–514.

Pozo M.J., Van Der Ent S., Van Loon L.C. & Pieterse C.M.J. (2008) Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytologist* **180**, 511–523.

Rani P.U. & Jyothsna Y. (2010) Biochemical and enzymatic changes in rice plants as a mechanism of defense. *Acta Physiologiae Plantarum* **32**, 695–701.

Raupach G.S. & Kloepper J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**, 1158–1164.

Ryu C.M., Kang B.R., Han S.H., Cho S.M., Kloepper J.W., Anderson A.J. & Kim Y.C. (2007) Tobacco cultivars vary in induction of systemic resistance against *Cucumber mosaic virus* and growth promotion by *Pseudomonas chlororaphis* O6 and its GACS mutant. *European Journal of Plant Pathology* **119**, 383–390.

Sarwar N., Zahid M.H., Haq C.I. & Jamil F.F. (2005) Induction of systemic resistance in chickpea against Fusarium wilt by seed treatment with salicylic acid and bion. *Pakistan Journal of Botany* **37**, 989–995.

Sato S., Nakamura Y., Kaneko T., Katoh T., Asamizu E., Kotani H. & Tabata S. (2000) Structural analysis of *Arabidopsis thaliana* chromosome 5. X. sequence features of the regions of 3,076,755 bp covered by sixty P1 and TAC clones. *DNA Research* **7**, 31–63.

Sobhy I.S., Erb M. & Turlings T.C.J. (2014) Plant strengtheners enhance parasitoid attraction to herbivore-damaged cotton via qualitative and quantitative changes in induced volatiles. *Pest Management Science* **7**, 686–693.

Townsend B.J., Poole A., Blake C.J. & Llewellyn D.J. (2005a) Antisense suppression of a (+)-delta-cadinene synthase gene in cotton prevents the induction of this defense response gene during bacterial blight infection but not its constitutive expression. *Plant Physiology* **138**, 516–528.

Townsend B.J., Poole A., Blake C.J. & Llewellyn D.J. (2005b) Antisense suppression of a (+)-δ-cadinene synthase gene in cotton prevents the induction of this defense response gene during bacterial blight Infection but not its constitutive expression. *Plant Physiology* **138**, 516–528.

Van Oosten V.R., Bodenhausen N., Reymond P., Van Pelt J.A., Van Loon L.C., Dicke M. & Pieterse C.M.J. (2008) Differential effectiveness of microbially induced resistance against herbivorous insects in Arabidopsis. *Molecular Plant-Microbe Interactions* **21**, 919–930.

War A.R., Paulraj M.G., War M.Y. & Ignacimuthu S. (2011) Jasmonic acid-mediated-induced resistance in groundnut (*Arachis hypogaea* L.) against *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Journal of Plant Growth Regulation* **30**, 512–523.

Wu G., Guo J.Y., Wan F.H. & Xiao N.W. (2010) Responses of three successive generations of beet armyworm, *Spodoptera exigua*, fed exclusively on different levels of gossypol in cotton leaves. *Journal of Insect Science* **10**, 165.

Xu Y.H., Wang J.W., Wang S., Wang J.Y. & Chen X.Y. (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-delta-cadinene synthase-A. *Plant Physiology* **135**, 507–515.

Zamioudis C. & Pieterse C.M.J. (2012) Modulation of host immunity by beneficial microbes. *Molecular Plant-Microbe Interactions* **25**, 139–150.

Zebelo S.A., Bertea C.M., Bossi S., Occhipinti A., Gnavi G. & Maffei M.E. (2011) *Chrysolina herbacea* modulates terpenoid biosynthesis of *Mentha aquatica* L. *Plos One* **6**.

Zehnder G., Kloepper J., Tuzun S., Yao C.B., Wei G., Chambliss O. & Shelby R. (1997a) Insect feeding on cucumber mediated by rhizobacteria-induced plant resistance. *Entomologia Experimentalis Et Applicata* **83**, 81–85.

Zehnder G., Kloepper J., Yao C.B. & Wei G. (1997b) Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth-promoting rhizobacteria. *Journal of Economic Entomology* **90**, 391–396.

Zehnder G., Yao C.B., Murphy J.F., Sikora E.R. & Kloepper J.W. (2000) Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth-promoting rhizobacteria. *Biocontrol* **45**, 127–137.

Zhang P.J., Zhu X.Y., Huang F., Liu Y., Zhang J.M., Lu Y.B. & Ruan Y.M. (2011) Suppression of jasmonic acid-dependent defense in cotton plant by the mealybug *Phenacoccus solenopsis*. *Plos One* **6**.

Zhang S.Z., Hua B.Z. & Zhang F. (2008) Induction of the activities of antioxidative enzymes and the levels of malondialdehyde in cucumber seedlings as a consequence of *Bemisia tabaci* (Hemiptera: Aleyrodidae) infestation. *Arthropod-Plant Interactions* **2**, 209–213.

Zheng S.J., van Dijk J.P., Bruinsma M. & Dicke M. (2007) Sensitivity and speed of induced defense of cabbage (*Brassica oleracea* L.): dynamics of BoLOX expression patterns during insect and pathogen attack. *Molecular Plant-Microbe Interactions* **20**, 1332–1345.

Zhou M.L., Zhang C.C., Wu Y.M. & Tang Y.X. (2013) Metabolic engineering of gossypol in cotton. *Applied Microbiology and Biotechnology* **97**, 6159–6165.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. qrtPCR amplicon sequences.